Virology Reports xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Virology Reports

journal homepage: www.elsevier.com/locate/virep

Mutations within ICP4 acquired during *in vitro* attenuation do not alter virulence of recombinant Marek's disease viruses *in vivo*

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ARTICLE INFO

Article history:
Received 14 June 2014
Received in revised form 17 November 2014
Accepted 23 November 2014
Available online xxxx

Keywords:
Marek's disease virus
Virulence
Attenuation
Vaccine
Horizontal transmission
ICP4
BAC

ABSTRACT

Marek's disease (MD) is a T-cell lymphoma of chickens caused by the oncogenic Marek's disease virus (MDV). MD is primarily controlled by live-attenuated vaccines generated by repeated *in vitro* serial passage. Previous efforts to characterize attenuated MDVs identified numerous mutations, particularly a convergence of high-frequency mutations around amino acids 60–63 within ICP4 (RS1), therefore, ICP4 was considered a candidate gene deserving further characterization. Recombinant MDVs were generated containing a single Q63H mutation or double Q63H + S1630P mutations. Despite the repetitive nature of mutations within ICP4, neither recombinant virus decreased virulence, although one mutant reduced *in vivo* replication and failed to transmit horizontally. Our results indicate that these mutations are insufficient to reduce disease incidence in infected birds, and suggest that variants in ICP4 do not directly alter virulence, but rather may enhance MDV replication rates *in vitro*, offering an explanation for the widespread occurrence of ICP4 mutations in a variety of attenuated herpesviruses.
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1. Introduction

Gallid herpesvirus 2, also commonly known as Marek's disease virus (MDV), is an oncogenic alphaherpesvirus of chickens. Afflicted birds display symptoms of Marek's disease (MD) including depression,

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http://dx.doi.org/10.1016/j.virep.2014.11.002
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cachexia, and paralysis due to viral-induced T-cell lymphomas that ultimately result in death. The primary mode of MD control has been *via* the use of vaccines, which has significantly reduced losses due to MD since the 1970s (Purchase and Okazaki, 1971). Despite the widespread use of vaccinal control of MD, virulent strains of MDV able to overcome vaccines have emerged, leading to the need for periodic introduction of new and more effective MD vaccines approximately every 10 years (Witter, 1997). Currently the most protective vaccine commercially available, CVI988/Rispens, has been in use in the United States since the 1990s. Concern regarding the potential for MD outbreaks in Rispens-vaccinated flocks has highlighted the importance for vaccine development (Gimeno, 2008). One common method for vaccine production has been the use of live attenuated viruses generated *via* repeated *in vitro* serial passage. Several MD vaccines, such as HPRSS-16 and Rispens, were generated by *in vitro* serial passage and provide testament to the utility of this process in vaccine development (Churchill et al., 1969; Rispens et al., 1972).

To better understand this process and identify candidate genes involved in attenuation, previously we serially passed and sequenced the complete genome of four attenuated MDV replicates (three Md5BAC-derived viruses and one Md5 strain) to identify candidate mutations and genes involved in attenuation (Hildebrandt et al., 2014). Several candidate mutations present within genes in the unique long (UL) or unique short (US) regions of the viral genome were characterized *via* recombinant viruses. Among the five point mutations tested, one recombinant virus revealed a single nucleotide mutation in UL5 (helicase–primase subunit) able to reduce disease incidence by 90% or more. Due to the additional complexity involved in mutating genes present within the two long and short repeat regions (TRL/IRL and TRS/IRS) in MDV, these candidate mutations were not initially tested. Therefore, the purpose of this study was the characterization of a top candidate gene identified within the repeat regions of the MDV genome.

ICP4, encoded by RS1, is an immediate early transcriptional regulator in herpesviruses located within the TRS/IRS region of MDV and a gene commonly mutated in all of the attenuated replicates. All four passed viruses had mutations within ICP4, with three viruses containing high frequency (80–100%) nonsynonymous mutations within amino acids 60–63 in ICP4. Three other high frequency (40% or higher) nonsynonymous mutations in ICP4 were also observed, including one at amino acid position 1630 (85% frequency). Due to these numerous high frequency, parallel mutations within the attenuated replicates, ICP4 was considered a candidate gene for attenuation. Using Red-mediated recombineering, we generated two recombinant viruses to examine how these mutations in ICP4 impacted virulence of MDV.

2. Results

2.1. In vivo trials

Disease incidences resulting from challenge with either Mut ICP4-1 (single ICP4 Q63H SNV) or Mut ICP4-2 (double Q63H and S1630P SNVs) were 100% and 95%, respectively. Compared to the parental virus that showed 84% MD incidence there was no significant difference between MD incidence of the parental Md5B40BAC-c1 and either recombinant ICP4 viruses (Fischer's exact test $p = 0.5320$), therefore, these

Table 1
Mutational primers for generation of recombinant ICP4 viruses.

Primer name	Sequence
ΔICP4-f	TTGTCTAAATTGTTATGAGGTTTGGGGACAATATTTATTGACTAGTCTtagggataacagggtaatcgattt
ΔICP4-r	CCACCATCTATTTGCGCCCCTCCTAAACCCTAAAAATGGACAACCCGCCTAGACTAGTCAATAAATATTGTCCCCAAACC TCATAACAATTTAGACAAgccagtgttacaaccaattaacc
Mut ICP4 Q63H-f	GCTAGCCGGACAATCGGGTACATACCGCCCCCACTCCAGTTACCATGGACA**t**CGCTCGCTTTCCAGCGGCCC tagggataacagggtaatcgattt
Mut ICP4 Q63H-r	GGCTATGGGCAGCGGGGACGGGGCCGCTGGAAAGCGAGCG**a**TGTCCATGGTAACTGGAGTGGGGGCGGT ATGgccagtgttacaaccaattaacc
Mut ICP4 S1630P-f	ACCCGATCAGCTGTTTCGAGGTCCTGGGCGTCCCCGACGC**c**CCACTTCATCATCCCAGTCTtagggataacagggtaatcgattt
Mut ICP4 S1630P-r	ATGGGAGATTTATCAGACGCAGACTGGGATGATGAAGTGG**g**GCGTCGGGGACGCCCAGGACgccagtgttacaaccaattaacc

Nucleotides in uppercase indicate the region of primer homologous to MDV for integration into the MDV genome while nucleotides in lowercase are regions complementary to the kanamycin cassette. The single lowercase nucleotide in bold and underlined indicates the point mutation altered for generation of recombinant viruses.

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Table 2
MD incidence of recombinant ICP4 mutant viruses.

Challenge virus	MD incidence in challenged birds		MD incidence in contact birds	
	# MD+ birds/Total birds	Percent MD	# MD+ birds/Total birds	Percent MD
Mut ICP4-1	10/10[a]	100	5/5	100
Mut ICP4-2	18/19[a]	95	0/5	0
Md5B40BAC-c1	16/19[a]	84	4/5	80

[a] Due to chick mortality during the first two weeks of age in isolators challenged with Mut ICP4-1, fewer total birds were available compared to Mut ICP4-2 and Md5B40BAC.

mutations did not reduce virulence of the viruses in infected birds (Table 2). While most challenged birds developed MD, the disease incidences in contact birds varied significantly between the two recombinant ICP4 viruses. Contact birds housed with the Mut ICP4-1 virus all developed MD, yet no contact birds mixed with birds challenged with the Mut ICP4-2 recombinant virus developed MD (Table 2).

While both recombinant viruses resulted in over 90% disease incidence, there was delayed mortality for birds challenged with the double Mut ICP4-2 recombinant virus compared to Mut ICP4-1 and Md5B40BAC-c1. Birds challenged with the single Mut ICP4-1 recombinant virus experienced survival comparable to the original Md5B40BAC-c1 (Fig. 1). On the other hand, the double Mut ICP4-2 virus exhibited lower mortality from week 4 onwards (Fig. 1), with approximately 20% of birds surviving the full length of the 12 week experiment compared to birds challenged with either Mut ICP4-1 or Md5B40BAC-c1 which were all euthanized by week 10.

Replication of MDV within the spleen of birds sacrificed at 7, 14 and 21 dpi as determined by qPCR showed a range among the three viruses (Fig. 2). Compared to Mut ICP4-1 and the wild-type Md5B40BAC virus, the double mutant Mut ICP4-2 recombinant virus showed lower levels of *in vivo* replication at 7 and 14 dpi, with significant differences in replication at day 14 (ANOVA p-value = 0.0136), though this difference was not observed at 21 dpi. Further pairwise comparisons of *in vivo* replication at day 14 showed significant differences in replication between Mut ICP4-1 and Md5B40BAC-c1, with differences between Mut ICP4-1 and Md5B40BAC-c1 falling short of the threshold for significance (*T*-test p = 0.0101 and p = 0.0557, respectively).

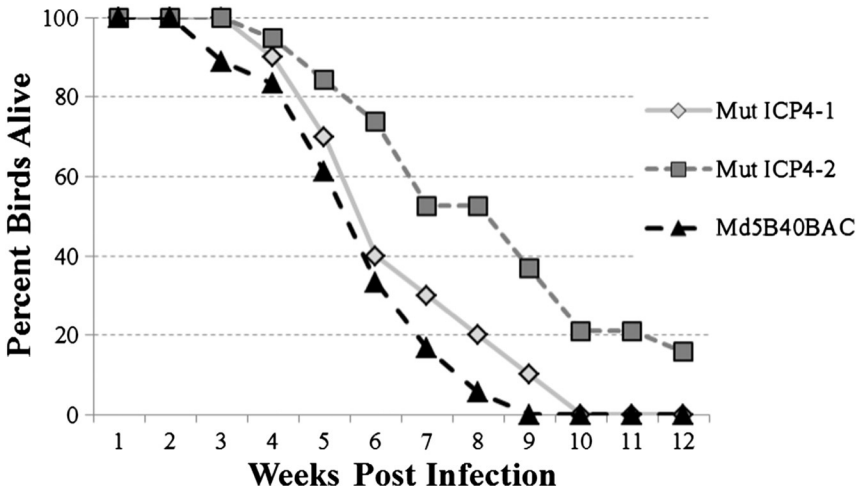

Fig. 1. Survival curves of birds challenged with recombinant MDV. Mortality of birds during the 12 week experiment until termination was compared for the two ICP4 mutant viruses relative to the parental Md5B40BAC virus.

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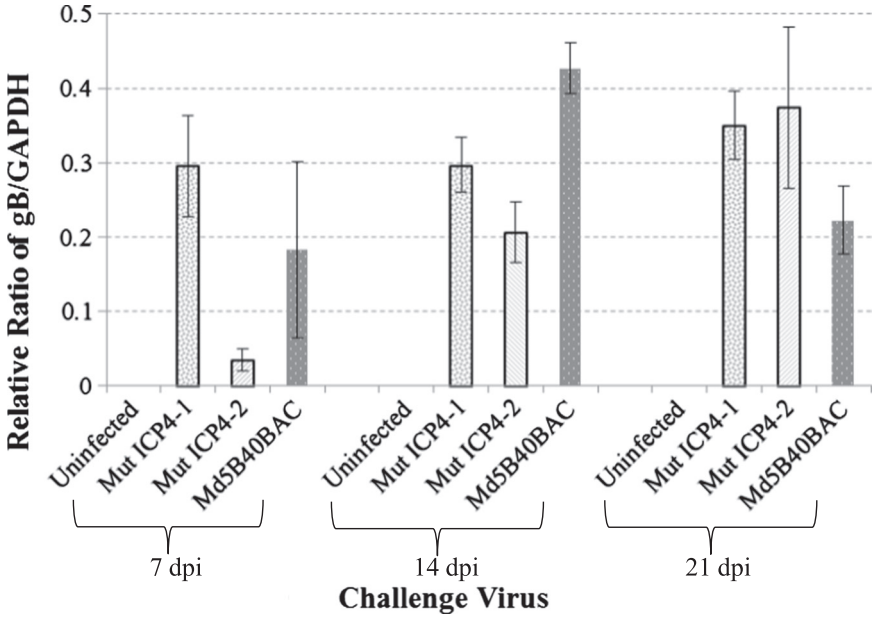

Fig. 2. Viral replication rates of birds challenged with recombinant MDV. *In vivo* replication of recombinant ICP4 MDV *versus* parental Md5B40BAC virus.

2.2. Homology of alphaherpesviruses' ICP4 protein sequence

Comparing the prototypical alphaherpesvirus (HSV-1) to the 20 alphaherpesviruses' sequences *via* pairwise alignment of HSV-1 to each respective species sequence showed a high degree of variation among ICP4 sequences between species. Amino acid sequence identity for ICP4 of the 20 species surveyed relative to HSV-1 ranged from approximately 15–71%, with an average shared sequence identity of 36% (Supp. Table 1). Alignment of HSV-1 to Mardivirus sequences show an average sequence identity of 17% among the three Mardivirus species, *versus* an average shared sequence identity of 39% for HSV-1 alignments with the non-Mardivirus species. Alignment of HSV-1 ICP4 and the virulent MDV ICP4 sequences revealed the lowest degree of shared sequence identity of approximately 15%.

Alignment of all 20 ICP4 sequences showed a high degree of variation among the alphaherpesviruses surveyed, not only in coding sequence but also in total length. The average length of ICP4 in the 20 alphaherpesviruses surveyed was approximately 1500 amino acids. However, the average length of ICP4 in the Mardivirus species infecting chicken (Gallid herpesvirus 2, Gallid herpesvirus 3 and Meleagrid herpesvirus 1) revealed significantly larger (2173 amino acids) ICP4 proteins, with Gallid herpesvirus 2 having the longest sequence of any herpesvirus species surveyed at 2323 amino acids (Supp. Table 1). The majority of these nearly 700 additional amino acids are found at the N terminus of ICP4 in Mardiviruses. Clustal alignment of the 20 herpesvirus species show that for the first 500 amino acids of MDV ICP4, the only species with homologous amino acids to this region are Gallid herpesvirus 3 and Meleagrid herpesvirus 1, both of which also belong to the Maridvirus genus (Supp. Fig. 1). Furthermore, the cluster of high frequency mutations within ICP4 identified in the attenuated MDV replicates around amino acids 60–63 occurs within this additional region that is unique relative to other alphaherpesviruses. Even among Mardiviruses that possess a longer ICP4, the region around amino acids 55–79 is exclusively found only in the virulent Gallid herpesvirus 2 species, preventing functional predictions for this mutated region based on homology to other species (Supp. Fig. 1).

The alignment of ICP4 sequences showed that the region surrounding the second mutation at amino acid 1630 is a position present in the majority of herpesvirus species, although there is considerable variation in the amino acids present at this position (Supp. Fig. 2). The MDV wild type serine is an amino acid seen in other herpesvirus species, such as Gallid herpesvirus 1 and both HSV-1 and HSV-2. The mutation of a serine

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to proline residue at position 1630 is predicted to be a tolerated change by programs such as SIFT (Kumar et al., 2009), as well as an evolutionarily acceptable amino acid considering that proline is commonly seen in several herpesvirus species, such as Equid herpesvirus 1, Suid herpesvirus 1, Psittacid herpesvirus 1, and the closely related Meleagrid herpesvirus 1 (Supp. Fig. 2). Based on the alignment to HSV-1, the MDV S1630P ICP4 mutation corresponds to roughly the same serine residue at position 683 in HSV-1. This position is expected to be a globular region within the third defined region of ICP4 in HSV-1. This position is also characterized as occurring within a functional domain implicated for nuclear localization (Wyrwicz and Rychlewski, 2007), yet the corresponding amino acid regions within MDV ICP4 do not appear to contain a known nuclear localization signal surrounding the mutated region in MDV ICP4 based on various prediction programs (NucPred, NLS Mapper and NLStradamus; data not shown).

Our phylogenetic tree constructed based on the complete sequence of ICP4 from the 20 herpesvirus species had an overall topology and grouping comparable to trees previously constructed using a compilation of several genes (McGeoch and Cook, 1994; McGeoch et al., 2000). However, despite the overall similarity for most genera between the two trees, there was a change in the relationship of the virulent Iltovirus, known as ILTV, and Gallid Mardiviruses for this tree generated based only on ICP4 compared to previously constructed trees. The resulting ICP4 tree has the most closely related sister taxa classified as the virulent Gallid herpesvirus-2 and ILTV, which are shown to split from the avirulent Gallid herpesvirus-3 and Meleagrid herpesvirus 1 lineage (Fig. 3). This yields classification of these three Mardiviruses and single Iltovirus species along lines of virulence, unlike previous studies that classify these species relationships based on genera lines. This relationship is dependent upon highly variable regions within ICP4, as trimming ICP4 sequences to only use regions known to be highly conserved among alphaherpesviruses, such as regions 2 and 4 (Wyrwicz and Rychlewski, 2007; McGeoch et al., 1986), results in a phylogenetic tree with the expected grouping of the Mardivirus and Iltovirus genera as separate branches (data not shown).

3. Discussion

During a previous *in vitro* serial passage of MDV to find candidate genes involved in attenuation, ICP4 was found to be mutated repeatedly in all attenuated MDV replicates sequenced (Hildebrandt et al., 2014). These attenuated viruses contained nonsynonymous mutations at amino acids 60, 62 and 63 at frequencies of 96%,

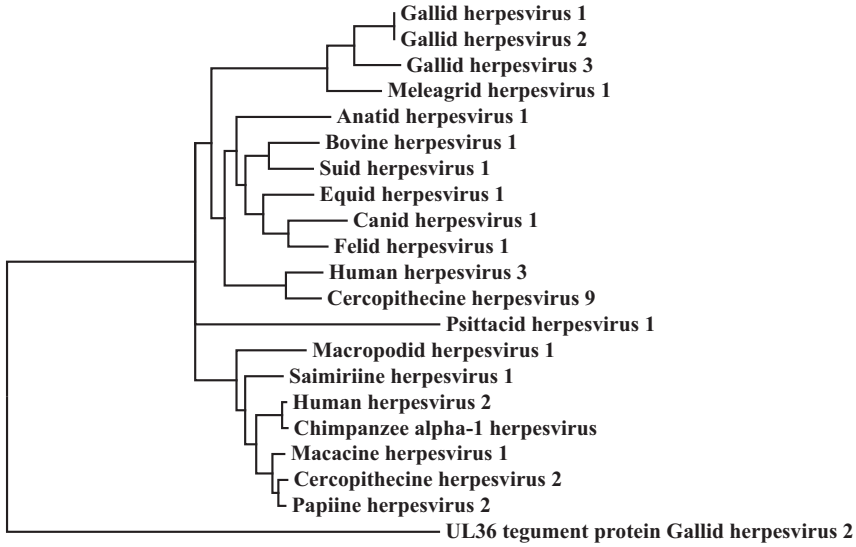

Fig. 3. Maximum likelihood phylogenetic tree based on the complete amino acid sequence of ICP4 from 20 herpesvirus species. Artificially rooted with the outgroup of UL36 amino acid sequence from Gallid herpesvirus 2.

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83% and 100%, respectively, in three of the four attenuated populations. Besides these clustered mutations, three more nonsynonymous mutations exceeding frequencies of 40% or more were also seen at other positions within ICP4 in the attenuated replicates. In addition to these serially passed Md5 replicates, mutations within ICP4 in attenuated MDV have been identified by other groups as well. During serial passage and sequencing of an attenuated 648A strain of MDV, three high frequency mutations within ICP4, two nonsynonymous and one silent mutation, were found within the attenuated strain (Spatz, 2010). Even during complete genome sequence analysis of the current "gold-standard" MD vaccine known as Rispens, mutations were found within ICP4 (Spatz et al., 2007). Studies including alphaherpesviruses besides MDV also show a similar trend of mutations within ICP4 in attenuated viruses. Complete genome sequencing of an attenuated and highly passed vaccine strain of the pseudorabies virus, Suid herpesvirus 1, identified 11 mutations within ICP4 that were unique to a vaccine strain compared to two sequenced virulent strains (Szpara et al., 2011). Furthermore, in the Oka vaccine strain of the varicella-zoster virus (VZV), also known as human herpesvirus 3 (HHV-3), a disproportionate number of mutations (15 with 8 of which were nonsynonymous) were found within ICP4 in the vaccine strain relative to the virulent parental virus (Gomi et al., 2002).

Despite evidence from a variety of attenuated alphaherpesviruses suggesting that mutations within ICP4 may be a causative factor driving attenuation of the virus, the two recombinant viruses tested containing high frequency candidate SNVs within ICP4 did not cause any reduction in virulence of MDV, although the Mut ICP4-2 recombinant virus did fail to transmit horizontally to contact birds. Recombinant MDV viruses that are virulent and induced MD in challenged birds but failed to transmit horizontally to cause MD in contact birds have been previously reported for mutations in UL44 (gC) and UL13 (protein kinase) (Jarosinski et al., 2007; Jarosinski and Osterrieder, 2010), as well as with a recombinant virus with a point mutation within LORF2 (Hildebrandt et al., 2014). One possible explanation for the failure of Mut ICP4-2 to infect contact birds is due to lower levels of *in vivo* replication at days 7 and 14, during the times traditionally classified as early cytolytic and latent phases of infection (Baigent and Davison, 2004). The lowest virus load among the three time points was seen during the transition between the early cytolytic phase to latency (7 dpi) for Mut ICP4-2. This could affect transmission of the recombinant virus because as chickens mature they develop an age-related resistance towards MD. Therefore, young chicks are most susceptible to MDV infection and due to a decrease or delay in shedding of virulent MDV by Mut ICP4-2, the potential for transmission and infection to older contact birds would decrease (Jarosinski et al., 2007). Alternatively, these mutations within ICP4 may impair replication within the feather follicles, leading to an inability to spread through shed feather dander. Despite the indication that mutations within ICP4 appear to be correlated with attenuation in a multitude of alphaherpesviruses, recombinant viruses containing point mutations within ICP4 show that mutations within ICP4 alone are not sufficient for attenuation in MDV. This conclusion is supported by experiments using the Oka vaccine strain of VZV in which transactivation of downstream promoters regulated by ICP4 was compared between mutated versions of ICP4 from the vaccine strain *versus* the parental virus. Cohrs et al. (2006) established that regulation and transcription of downstream promoters between the mutated and wild-type ICP4 were comparable, leading to their conclusion that mutations within ICP4 alone are not sufficient to cause attenuation.

Mutations within the VZV Oka vaccine strain did not affect transactivation of viral promoters, but it is known that ICP4 contains different regions with specific functions in addition to transactivation, such as DNA binding, nuclear localization, and regulation of late genes (Wyrwicz and Rychlewski, 2007). Therefore, we sought to computationally determine what function observed mutations within MDV ICP4 may impact, particularly those within amino acids 60–63, which contained mutations in three completely attenuated MDV replicates *via* sequence comparisons to other alphaherpesviruses. Alignment of the complete amino acid sequence of ICP4 revealed significant variation among alphaherpesviruses, which has previously been described. It is known that certain regions of ICP4, such as regions 2 and 4, are highly conserved among herpesviruses, while other regions are not particularly well conserved among species, such as regions 1, 3 and 5 (Wyrwicz and Rychlewski, 2007; McGeoch et al., 1986). Specifically, comparisons among genera show that Mardiviruses have a significantly longer ICP4 of approximately 2173 amino acids in length compared to the average length of 1500 amino acids for non-Mardiviruses. Furthermore, in our attenuated MDV replicates containing several high frequency mutations around amino acids 60–63, these mutations occur within a region found uniquely in MDV yet absent in other closely related Mardiviruses. Considering that MDV is not only virulent, but is also an oncogenic virus, unlike all other alphaherpesviruses which do not cause tumors, it is difficult to predict what role these unique regions in MDV ICP4 provide during the life cycle of

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this oncogenic herpesvirus, particularly in light that closely related apathogenic Mardiviruses lack this commonly mutated region of ICP4.

The second mutation at amino acid 1630 occurs at a position present within the 20 herpesvirus species surveyed unlike the first mutation at amino acid 63. The mutation of a serine residue to a proline at position 1630 appears to be an evolutionary accepted change, with proline commonly found in several other species of herpesviruses. Despite this apparently tolerated amino acid change, it is only with the addition of this second mutation at position 1630 in conjunction with the mutation at amino acid 63 that changes in horizontal transmission were observed. The role for this second mutation at position 1630 is difficult to predict due to the high degree of variation among herpesvirus species for amino acids within this designated region 3 of ICP4, which has been shown to be less highly conserved than regions 2 and 4 (Wyrwicz and Rychlewski, 2007; McGeoch et al., 1986), leading to a poorer understanding of functional roles for diverse regions such as region 3. Analysis of MDV's ICP4 primary structure *via* software designed for modeling and prediction of protein structure, such as for MDV ICP4 sequences predict that the regions of MDV ICP4 containing the two mutations engineered are found within disordered, coiled regions of the protein but due to lack of MDV ICP4 structural predictions, little data is available for hypothesizing the roles for the observed mutated regions.

Due to limitations of comparisons of ICP4 for specific amino acid positions among the herpesviruses surveyed, further comparison of the entire ICP4 sequence *via* phylogenetic analysis of the 20 alphaherpesvirus species based on the complete ICP4 amino acid sequence showed a discrepancy between the relationship of Mardiviruses and Iltoviruses of chicken compared to traditional trees based on compilation of data involving multiple genes. Previous studies have classified the three Mardiviruses (Melaeagrid herpesvirus 1, Gallid herpesvirus 2 and Gallid herpesvirus 3) collectively in one group known as α3 viruses, while the Iltovirus, Gallid herpesvirus 1 (ILTV), was classified in a separate group designated α4 based on genera lines, as would be expected (McGeoch et al., 2000). Instead, based on ICP4 sequences, these relationships are no longer in line with genera classification, but show closer homology between the virulent viruses of Gallid herpesvirus 2 and ILTV now grouped as sister taxa, despite belonging to different genera. Thus, it appears that there may be a greater commonality between evolutionary pressure affecting the sequence of ICP4 within virulent viruses which affect chickens compared to other Mardi and Iltoviruses.

Collectively, this data suggests that mutations within ICP4 may play a supporting role in attenuation, cooperatively with the addition of other mutations that directly reduce virulence. Due to a selective advantage for viruses able to replicate faster during serial passage, mutations that impact *in vitro* replication would be expected to occur within attenuated viruses despite the fact that those mutations themselves are not causative for the loss of virulence resulting from serial passage. This would result in mutations that impact *in vitro* replication to occur collectively with mutations that reduce virulence *in vivo* and are the causative mutation for attenuation and the loss of virulence. One such potential scenario for the attenuated MDV viruses sequenced involves a point mutation within the UL5 helicase–primase gene identified at 65% in an attenuated MDV viral population that likely could have occurred concurrently within the same viral genome containing the ICP4 mutation G62V present at 83% in the same population. This mutation in UL5 reduced virulence by nearly 90% (Hildebrandt et al., 2014), therefore, it may be postulated that mutations within ICP4, which previously have been proposed to provide a selective advantage during *in vitro* growth (Gomi et al., 2002), could result in attenuated viruses generated after repeated serial passage due to combination with additional mutations that alter virulence, such as the UL5 mutation.

Despite the large body of examples correlating mutations within ICP4 with attenuation in alphaherpesviruses, it remains unknown what function these mutations may impart to attenuated viruses. Without further characterization it would be difficult to further postulate what role these additional 700 amino acids confer in MDV relative to other alphaherpesviruses, or understand the impact of the numerous mutations within ICP4 seen not only within attenuated MDV, but among other alphaherpesviruses as well.

4. Conclusions

Despite showing multiple nonsynoymous mutations at high frequency in attenuated MDVs, neither Q63H nor S1630P mutations were sufficient to reduce viral virulence and are not causative factors for the loss of virulence during *in vitro* serial passage. Therefore, recombinant MDVs with only defined mutations within ICP4 are not likely to be viable candidates as MD vaccines.

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5. Materials and methods

5.1. Recombinant viruses and tissue culture

ICP4 mutations were incorporated into Md5B40BAC, the pBeloBAC11 clone containing the entire MDV (Md5 strain) genome, using Red-mediated recombineering (Jarosinski et al., 2007; Tischer et al., 2006; Niikura et al., 2011). To generate recombinant viruses containing the desired single nucleotide variant (SNV) within both copies of ICP4, one copy of ICP4 was first deleted using the ΔICP4 primer set (Table 1) based on the process of manipulating genes within repeat regions described by Engel et al. (2012). Point mutations were then incorporated within the remaining copy of ICP4 *via* additional rounds of recombineering using the primer sets Mut ICP4 Q63H and Mut ICP4 S1630P. The resulting mutants, designated as Mut ICP4-1 and Mut ICP4-2, both contained the mutation Q63H, while the latter also had the additional SNV S1630P. The Q63H mutation was fixed 100% in one of the serially passed attenuated replicates during our previous study (Hildebrandt et al., 2014), while the S1630P mutation was the second highest frequency ICP4 mutation within the same replicate present at 85%. All introduced mutations were confirmed *via* Sanger sequencing before transfecting the mutated BACs into duck embryo fibroblasts (DEF) *via* the calcium phosphate method (Moriuchi et al., 1992) and viral stocks passed through DEFs for four passages to amplify viral stocks. Additionally, mutations in the generated viral stocks were confirmed by Sanger sequencing. Viral stocks were then titrated on DEF cells to quantify the number of plaque forming units (PFU) in frozen viral stocks.

5.2. In vivo characterization of mutant viruses

Maternal antibody negative, ADOL $15I_5 \times 7_1$ day old chicks were challenged intraabdominally with 500 PFU of each recombinant MDV or the original Md5B40BAC-c1 as a positive control, and housed in Horsfall–Bauer (HB) isolators for 12 weeks. Uninfected birds were housed as negative controls in a separate isolator. All surviving birds, or those that were moribund during the course of the experiment, were terminated and examined *via* necropsy to determine disease incidence. To determine horizontal transmission, uninfected contact birds were housed in the same HB unit with challenged birds. Five infected birds were removed for sampling at 7, 14 and 21 days post infection (dpi) to collect spleen tissues to quantify *in vivo* replication of viruses *via* qPCR comparing the relative quantity of the MDV gB gene to chicken GAPDH gene using a Taqman Fast Universal PCR kit (Applied Biosciences; Foster City, CA) and primers described by Gimeno et al. (2008). All *in vivo* bird trials were approved by the USDA, Avian Disease and Oncology Laboratory Animal Care and Use Committee (ACUC). Based on ACUC guidelines established and approved by the ADOL ACUC (April 2005) and the Guide for the Care and Use of Laboratory Animals by the Institute for Laboratory Animal Research (2011), procedures for bird housing and care were followed throughout the duration of the experiment.

5.3. Sequence comparison of ICP4 among alphaherpesviruses

The complete amino acid sequence of the immediate early transcriptional regulator gene ICP4 from 20 different species of alphaherpesviruses was obtained from the NCBI database. All 20 sequences were aligned using ClustalW (Larkin et al., 2007) and a maximum likelihood phylogenic tree constructed in Mega6 (Tamura et al., 2013), while pairwise comparisons for each of the 20 ICP4 sequences relative to herpes simplex virus 1 (HSV-1) ICP4 (GenBank accession no. AGZ01922.1) were aligned *via* the UniProt protein resources database (The UniProt Consortium, 2014) to calculate the percentage of shared sequence identity among ICP4 sequences.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.virep.2014.11.002.

Acknowledgments

We thank Laurie Molitor for assistance in birdwork, Lonnie Milam for his exemplary technical advice, and Spencer Jackson for Histopaque isolation of PBLs, DNA extraction and qPCR sample preparation. This project was supported in part by National Research Initiative Competitive Grant no. 2010-65119-20505 from the USDA National Institute of Food and Agriculture.

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Please cite this article as: Hildebrandt, E., et al., Mutations within ICP4 acquired during *in vitro* attenuation do not alter virulenc..., Virol Rep (2014), http://dx.doi.org/10.1016/j.virep.2014.11.002